

## Block of Sodium Channels by Divalent Mercury: Role of Specific Cysteiny Residues in the P-Loop Region

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**ABSTRACT** Divalent mercury ( $\text{Hg}^{2+}$ ) blocked human skeletal  $\text{Na}^+$  channels (hSkM1) in a stable dose-dependent manner ( $K_d = 0.96 \mu\text{M}$ ) in the absence of reducing agent. Dithiothreitol (DTT) significantly prevented  $\text{Hg}^{2+}$  block of hSkM1, and  $\text{Hg}^{2+}$  block was also readily reversed by DTT. Both thimerosal and 2,2'-dithiodipyridine had little effect on hSkM1; however, pretreatment with thimerosal attenuated  $\text{Hg}^{2+}$  block of hSkM1. Y401C+E758C rat skeletal muscle  $\text{Na}^+$  channels ( $\mu 1$ ) that form a disulfide bond spontaneously between two cysteines at the 401 and 758 positions showed a significantly lower sensitivity to  $\text{Hg}^{2+}$  ( $K_d = 18 \mu\text{M}$ ). However, Y401C+E758C  $\mu 1$  after reduction with DTT had a significantly higher sensitivity to  $\text{Hg}^{2+}$  ( $K_d = 0.36 \mu\text{M}$ ) than wild-type hSkM1. Mutants C753A $\mu 1$  ( $K_d = 8.47 \mu\text{M}$ ) or C1521A  $\mu 1$  ( $K_d = 8.63 \mu\text{M}$ ) exhibited significantly lower sensitivity to  $\text{Hg}^{2+}$  than did wild-type hSkM1, suggesting that these two conserved cysteiny residues of the P-loop region may play an important role in the  $\text{Hg}^{2+}$  block of the hSkM1 isoform. The heart  $\text{Na}^+$  channel (hH1) was significantly more sensitive to low-dose  $\text{Hg}^{2+}$  ( $K_d = 0.43 \mu\text{M}$ ) than was hSkM1. The C373Y hH1 mutant exhibited higher resistance ( $K_d = 1.12 \mu\text{M}$ ) to  $\text{Hg}^{2+}$  than did wild-type hH1. In summary,  $\text{Hg}^{2+}$  probably inhibits the muscle  $\text{Na}^+$  channels at more than one cysteiny residue in the  $\text{Na}^+$  channel P-loop region.  $\text{Hg}^{2+}$  exhibits a lower  $K_d$  value ( $<1.23 \mu\text{M}$ ) for inhibition by forming a sulfur-Hg-sulfur bridge, as compared to reaction at a single cysteiny residue with a higher  $K_d$  value ( $>8.47 \mu\text{M}$ ) by forming sulfur-Hg<sup>+</sup> covalently. The heart  $\text{Na}^+$  channel isoform with more than two cysteiny residues in the P-loop region exhibits an extremely high sensitivity ( $K_d < 0.43 \mu\text{M}$ ) to  $\text{Hg}^+$ , accounting for heart-specific high sensitivity to the divalent mercury.

## INTRODUCTION

Sulfhydryl groups of cysteiny residues of peptides and proteins are the most reactive of all amino acid side chains under physiological conditions (Kenyon and Bruice, 1977). The function of cysteine-containing proteins often critically depends on the oxidative state of one or more of the protein's sulfhydryl groups (Ziegler, 1985; Walters and Gilbert, 1986; Creighton, 1977). Sulfhydryl groups of cysteiny residues also play a key role in regulating the functions of the ionic channels. There is evidence that the formation of disulfide (S-S) bonds alters channel conformation, producing changes in the permeability and gating kinetics of ion channels: 1) the opening of  $\text{Ca}^{2+}$  release channels on the sarcoplasmic reticulum membrane by S-S bonding (Salama et al., 1992), 2)  $\text{Ca}^{2+}$  channel block by sulfhydryl-oxidizing agents (Chiamvimonvat et al., 1995), 3) the activation of ATP-sensitive  $\text{K}^+$  channel (Coetzee et al., 1995; Tanaka et al., 1998) and nonselective cation channel (Jabr and Cole, 1995) by thiol compounds, and 4) the occlusion of the  $\text{Na}^+$

channel pore by S-S bridge formation (Benitah et al., 1997). These previous findings indicate that the structure and function of cysteine-containing ion channel proteins are critically dependent on the oxidation state of the sulfhydryl group. Because known amino acid sequences of the pore-forming region ( $\alpha$  subunit) of the  $\text{Na}^+$  channel indicate the presence of multiple cysteiny residues within the putative pore and other functional regions (Fozzard and Hanck, 1996), sulfhydryl modifications may be expected to affect the permeability or gating kinetics of the  $\text{Na}^+$  channel in various ways. The thiol-avid group IIB divalent cations, including  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$ , have been demonstrated to block the cardiac tetrodotoxin-insensitive  $\text{Na}^+$  channel by binding to one or more free sulfhydryl groups of critical cysteine residues (metal coordinate sites) within the permeation pathway of the channel protein (Ravindran et al., 1991; Schild et al., 1991; Schild and Moczydlowski, 1991, 1994; Doyle et al., 1993). Moreover, paired cysteine substitution experiments indicate that oxidation reactions forming a disulfide bond in the P-loop region can also determine the accessibility of the metal coordinate site (Benitah et al., 1997; Tomaselli, 1997). However, it has been reported that a sulfhydryl-oxidizing agent, 2,2'-dithiodipyrimidine (DTDP), produced no detectable changes in  $\text{Na}^+$  channel behavior (Chiamvimonvat et al., 1995). Therefore, the exact manner in which the sulfhydryl-oxidizing agents react with cysteiny sulfhydryl groups of  $\text{Na}^+$  channels is still unclear.

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In the present study, we examined the effects of sulfhydryl-oxidizing and reducing agents on the function of heterologously expressed  $\alpha$  subunits of wild and mutant skeletal muscle-type or heart-type Na<sup>+</sup> channels. The aim of the present study was to determine 1) the kind of sulfhydryl-oxidizing agents that affect the  $\alpha$  subunit of the Na<sup>+</sup> channel, 2) whether a sulfhydryl reducing agent can protect the  $\alpha$  subunit of the Na<sup>+</sup> channel from modification by oxidizing agents, 3) the molecular mechanism involved in the block of Na<sup>+</sup> channel by Hg<sup>2+</sup>, 4) whether the sensitivity of hSkM1 to Hg<sup>2+</sup> is different from that of hH1, and 5) the molecular mechanism underlying the isoform-specific sensitivity to Hg<sup>2+</sup>.

## MATERIALS AND METHODS

### Cell culture and transfection conditions

The vector pRC/CMV (Invitrogen, San Diego, CA) was used as the expression vector for hSkM1 and hH1 cDNAs. hSkM1 and hH1 cDNAs described elsewhere (Makita et al., 1996) were used. The mutant C753A, C1521A, and Y401C+E758C rat skeletal Na<sup>+</sup> channel  $\mu$ 1 cDNAs were provided by Dr. Gordon F. Tomaselli. COS7 cells were routinely maintained in Dulbecco's modified Eagle medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin and streptomycin. Cell cultures were kept at 37°C in a 5% CO<sub>2</sub> incubator. Cells grown on glass coverslips in 30-mm dishes were cotransfected with hSkM1,  $\mu$ 1 or hH1 cDNA and green fluorescence protein cDNA (Invitrogen) as a marker to identify the transfected cells with the calcium phosphate method (Hisatome et al., 1998). After 48 h, transiently transfected cells were visualized with fluorescent light, and subsequently electrophysiological recordings were performed, using the cells expressing green fluorescence protein.

### Mutagenesis

We performed site-specific mutagenesis to construct the mutant C373Y hH1, substituting Cys<sup>373</sup> for Tyr<sup>373</sup> (Hisatome et al., 1998).

### Electrophysiological recordings in cultured cells

Whole-cell voltage-clamp experiments were performed at 22°C as described (Bennett et al., 1993). The external solution had the following composition (mM): 140 NaCl, 5.0 CsCl, 1.0 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 HEPES, and 10 glucose (pH 7.4 with NaOH). Patch-clamp electrodes were filled with a solution of the following composition (mM): 90 CsF, 10 CsCl, 10 EGTA, 10 NaF, 2 MgCl<sub>2</sub>, and 10 HEPES (pH 7.4 with CsOH). To test the effect of sulfhydryl-oxidizing agents on the Na<sup>+</sup> channel, the current was elicited with a depolarizing pulse of -20 mV for 30 ms from a holding potential (HP) of -120 mV at 0.3 Hz before and after administration of sulfhydryl-oxidizing agents.

### Chemical agents for sulfhydryl modification

Reactive disulfide compounds with a pyridyl ring adjacent to the disulfide bond, such as 2,2'-dithiodipyridine (DTDP), oxidize appropriately positioned free sulfhydryl groups (especially via thiol-disulfide exchange reaction) with a hydrophobic modification, leading to the production of mixed disulfide bonds with protein and stoichiometric production of thio-pyridone. Thimerosal is a mercury compound that also oxidizes appropriate

positioned free sulfhydryl groups, but with a hydrophilic modification. Hg<sup>2+</sup> is a hydrophilic sulfhydryl-oxidizing agent that is capable of coordinated ligation of two cysteinyl residues (sulfur-Hg-sulfur) or coordinated binding to a single cysteine residue (sulfur-Hg<sup>+</sup>). 1,4-Dithiothreitol (DTT) was used to reduce disulfide bonds. This reagent has a very low redox potential, leading to a reaction yielding intramolecular disulfide bonds and free sulfhydryl groups on the channel proteins.

### Data analysis

The concentration dependence of Hg<sup>2+</sup>-induced block was fitted by the following equation:

$$I_B/I_C = 1/[1 + (K_d/D)^h] \quad (1)$$

where  $I_B$  is the blocked current component at a Hg<sup>2+</sup> concentration of  $D$ ,  $I_C$  is the control current in the absence of Hg<sup>2+</sup>, and  $K_d$  and  $h$  represent the dissociation constant and Hill coefficient, respectively.

Pooled data are presented as mean  $\pm$  SD. The statistical analysis was performed using Student's *t*-test and analysis of variance, with a value of  $p < 0.05$  considered significant.

## RESULTS

### Effects of the hydrophilic sulfhydryl-oxidizing agent Hg<sup>2+</sup> on hSkM1

Fig. 1, *A* and *B*, shows the dose-dependent effects of Hg<sup>2+</sup> on peak inward Na<sup>+</sup> current of hSkM1 during the control period, superfusion with Hg<sup>2+</sup> (1 and 3  $\mu$ M), washout with normal Tyrode's solution, and finally superfusion with 10  $\mu$ M Hg<sup>2+</sup>. Hg<sup>2+</sup> gradually reduced the amplitude of the hSkM1 from 1.5 (*a* in Fig. 1, *A* and *B*) to 0.9 nA (*b* in Fig. 1, *A* and *B*) by the administration of 1  $\mu$ M Hg<sup>2+</sup> and from 0.9 to 0.3 nA by treatment with 3  $\mu$ M Hg<sup>2+</sup>. The simple washout with normal Tyrode's solution for 3 min did not change the amplitude of hSkM1. Hg<sup>2+</sup> at 10  $\mu$ M blocked the hSkM1 immediately and completely (*c* and *d* in Fig. 1, *A* and *B*). Fig. 1 *C* indicates the dependence of this blockade on the concentration of Hg<sup>2+</sup>, obtained in four to six experiments. Hg<sup>2+</sup> started to block hSkM1 from 0.3  $\mu$ M and markedly blocked it at 1.0  $\mu$ M. The fitted  $K_d$  value and Hill coefficient for Hg<sup>2+</sup> were 0.964  $\mu$ M and 1.842, respectively. These results suggest that the hydrophilic sulfhydryl-oxidizing compound Hg<sup>2+</sup> blocked hSkM1 in a concentration-dependent fashion and Hg<sup>2+</sup>-modified sulfhydryls were stable after washout, which is similar to previous results (Islam et al., 1993; Ruppersberg et al., 1991).

Fig. 2 shows the effects of Hg<sup>2+</sup> at 3  $\mu$ M on the current-voltage relationship of hSkM1 obtained from seven experiments. Hg<sup>2+</sup> at 3  $\mu$ M significantly blocked hSkM1 without changes in threshold and peak potentials.

### Block of hSkM1 by HgCl<sub>2</sub> was due to the oxidation of free sulfhydryl groups

Although the effects of sulfhydryl oxidation by Hg<sup>2+</sup> were quite stable in the absence of reducing agent, this effect can

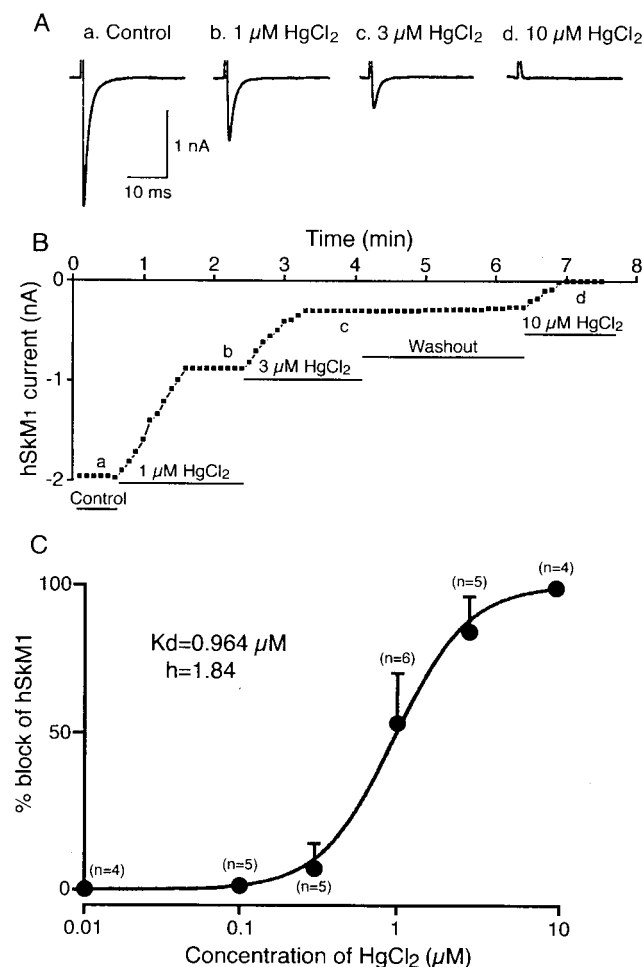


FIGURE 1 Concentration-dependent effects of the hydrophilic sulfhydryl-oxidizing agent  $\text{Hg}^{2+}$  on hSkM1. (A and B) Representative traces indicating the original currents in the control period (a) and in the presence of 1  $\mu\text{M}$  (b), 3  $\mu\text{M}$  (c), and 10  $\mu\text{M}$  (d)  $\text{Hg}^{2+}$ . The squares (a–d) plotted along the time in B correspond to the individual amplitudes of the current in A. (C) The mean  $\pm$  SD values of the % block of hSkM1 by  $\text{Hg}^{2+}$  at various concentrations (0.1–10  $\mu\text{M}$ ) in four to six experiments. The solid line shows the best fit by Eq. 1 with the optimal values of the parameters.

be reversed by using the sulfhydryl-reducing agent DTT. Fig. 3 A shows hSkM1 currents during superfusion with 10 mM DTT (part a), during superfusion with 3  $\mu\text{M}$   $\text{Hg}^{2+}$  (part b), and finally after superfusion with 10  $\mu\text{M}$   $\text{Hg}^{2+}$  (part c). In the presence of 10 mM DTT reducing agent, 3 and 10  $\mu\text{M}$   $\text{Hg}^{2+}$  did not reduce hSkM1. These results indicate that reduction with DTT protected the hSkM1 from the blocking effects of  $\text{Hg}^{2+}$ . Fig. 3 B illustrates the relationship between the ratio of the block of hSkM1 and the concentrations of  $\text{Hg}^{2+}$  with or without reduction with 10 mM DTT obtained from three to six individual experiments. The amount of block of hSkM1 by  $\text{Hg}^{2+}$  after treatment with 10 mM DTT was significantly smaller than that in the absence of DTT.

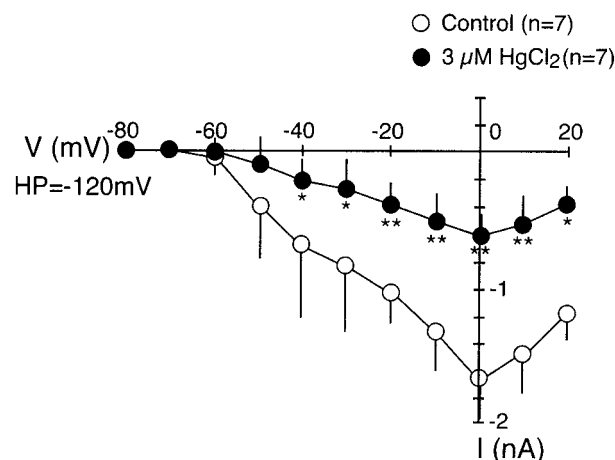


FIGURE 2 Effects of  $\text{HgCl}_2$  on the current-voltage relationship of hSkM1. A summary of the current-voltage relationship of hSkM1 in the absence and presence of 3  $\mu\text{M}$   $\text{Hg}^{2+}$  from seven experiments is shown. The data are the mean  $\pm$  SD.  $\text{Hg}^{2+}$  blocked hSkM1 without changes in either the threshold or peak potentials.

Fig. 3, C and D, shows the time course of hSkM1 during control, during superfusion with 3.0  $\mu\text{M}$   $\text{Hg}^{2+}$ , and after switching to 10 mM DTT alone.  $\text{Hg}^{2+}$  at 3.0  $\mu\text{M}$  reduced hSkM1 by 83% from 0.6 (Fig. 3 C a) to 0.1 nA (Fig. 3 C b); subsequently, 10 mM DTT partially reversed the  $\text{Hg}^{2+}$ -induced block of hSkM1, as shown (Fig. 3 C b–d), indicating that a substantial fraction ( $\sim 50\%$ ) of  $\text{Hg}^{2+}$ -induced block of hSkM1 is accessible to reversal by DTT. These results may be explained by DTT-dependent  $\text{Hg}^{2+}$ -reacted SH groups or by disulfide bond reaction.

### Sulfhydryl-oxidizing agent thimerosal and DTDP are ineffective in blocking the $\text{Na}^+$ current but modify the $\text{Hg}^{2+}$ -induced block of $\text{Na}^+$ current

Fig. 4 A shows the effects of 50  $\mu\text{M}$  thimerosal on hSkM1. Thimerosal alone did not reduce the amplitude of hSkM1. Fig. 4 B is a summary of the effects of 50  $\mu\text{M}$  thimerosal ( $n = 4$ ), 50  $\mu\text{M}$  DTDP ( $n = 4$ ), or 1  $\mu\text{M}$   $\text{Hg}^{2+}$  ( $n = 6$ ) on hSkM1 and the effects of  $\text{Hg}^{2+}$  (1–10  $\mu\text{M}$ ) in the presence of 50  $\mu\text{M}$  thimerosal. Thimerosal or DTDP alone did not block hSkM1, but  $\text{Hg}^{2+}$  blocked it significantly. In contrast, in the presence of 50  $\mu\text{M}$  thimerosal, the blocking effect of various concentrations of  $\text{Hg}^{2+}$  on hSkM1 ( $7 \pm 14.7\%$  at 1  $\mu\text{M}$   $\text{Hg}^{2+}$ ,  $6.3 \pm 28.2\%$  at 3  $\mu\text{M}$   $\text{Hg}^{2+}$ ,  $-23 \pm 7.6\%$  at 10  $\mu\text{M}$   $\text{Hg}^{2+}$ ;  $n = 4$ ;  $p < 0.05$ ) were significantly less than that in the absence of thimerosal ( $-58.4 \pm 14.0\%$  at 1  $\mu\text{M}$   $\text{Hg}^{2+}$ ,  $-84.8 \pm 14.2\%$  at 3  $\mu\text{M}$   $\text{Hg}^{2+}$ ,  $-100\%$  at 10  $\mu\text{M}$   $\text{Hg}^{2+}$ ;  $n = 4$ –5). These results suggest that thimerosal can access a reactive site and produce the simple oxidation of free sulfhydryl groups without blocking hSkM1 current.

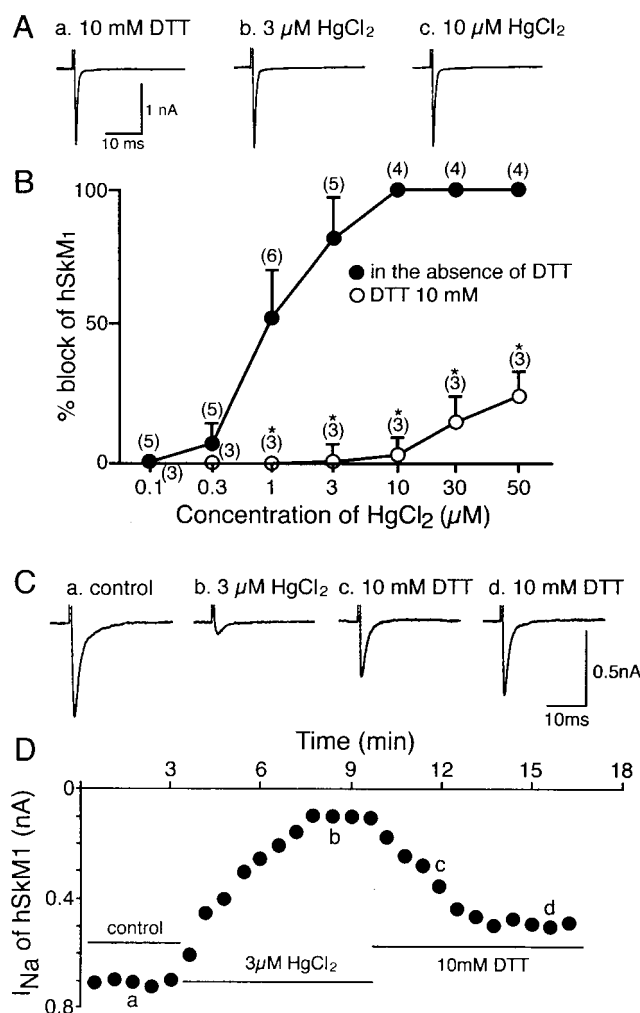


FIGURE 3 Pre- or posttreatment with DTT abolished the block of hSkM1 by Hg<sup>2+</sup>. (a) Representative current traces for hSkM1 in the presence of 10 mM DTT alone (a), in the presence of 3  $\mu$ M Hg<sup>2+</sup> (b), and in the presence of 10  $\mu$ M Hg<sup>2+</sup> (c). (B) Open and closed circles show the mean  $\pm$  SD values of the % block of hSkM1 induced by Hg<sup>2+</sup> (0.1–50  $\mu$ M) in the absence and presence of 10 mM DTT, respectively, in three to six experiments. (C and D) Closed circles indicate the representative amplitudes of the hSkM1 in control, in the presence of 3  $\mu$ M Hg<sup>2+</sup> alone, and in the presence of 10 mM DTT alone. The original current traces in a–d correspond to the amplitudes of a–d.

### Hg<sup>2+</sup> also blocks Na<sup>+</sup> channel $\alpha$ subunits by forming the coordinated ligation between a pair of cysteinyl residues

To prove that closely spaced free sulfhydryl groups can form a bridged coordination site for Hg<sup>2+</sup>, we studied the effects of Hg<sup>2+</sup> on current through the double mutant Y401C+E758C  $\mu$ 1 rat skeletal muscle Na<sup>+</sup> channel  $\alpha$  subunits. As shown in Fig. 5 A (a), current through the Y401C+E758C  $\mu$ 1 increased during exposure to reduced DTT of 1 mM. On average ( $n = 5$ ), 1 mM DTT reversibly and significantly augmented the peak Na<sup>+</sup> current by  $47 \pm$

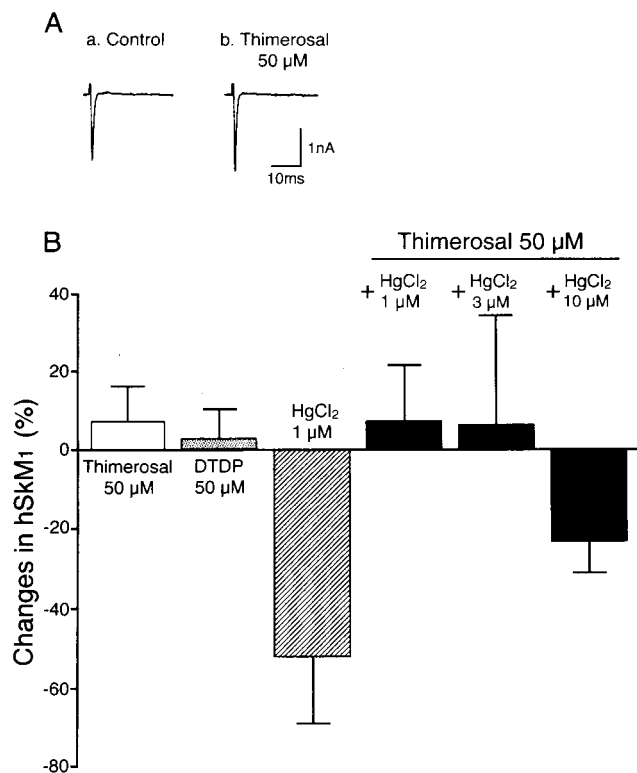
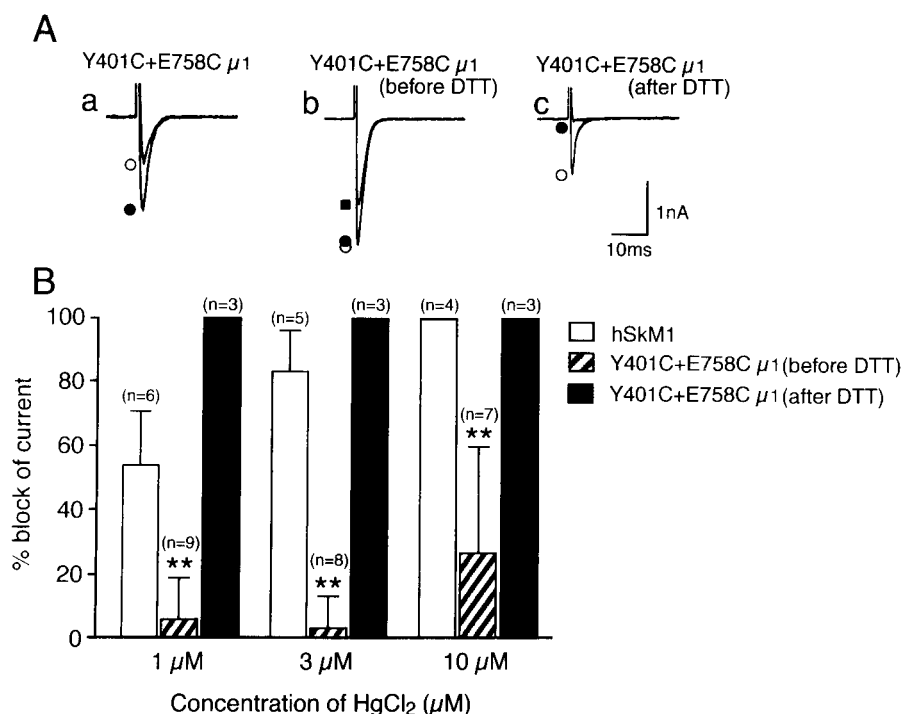


FIGURE 4 Effects of thimerosal and DTDP on hSkM1. (A) Representative current traces indicating the original current of hSkM1 in the control (a) and in the presence of 50  $\mu$ M thimerosal (b). (B) Each column plus bar indicates the mean  $\pm$  SD value of the % block of hSkM1 induced by 50  $\mu$ M thimerosal, 50  $\mu$ M DTDP, 1  $\mu$ M Hg<sup>2+</sup>, 1  $\mu$ M Hg<sup>2+</sup> + 50  $\mu$ M thimerosal, 3  $\mu$ M Hg<sup>2+</sup> + 50  $\mu$ M thimerosal, and 10  $\mu$ M Hg<sup>2+</sup> + 50  $\mu$ M thimerosal obtained from four to six experiments each.

17% ( $p < 0.01$ ). The augmentation of current by reducing agents implies that a disulfide bond forms spontaneously between cysteines at 401 and 758 positions, bridging the P-loops of domains I and II and partially occluding the pore. The reduction of current through Y401C+E758C  $\mu$ 1 by either 3 or 10  $\mu$ M Hg<sup>2+</sup> (Fig. 5 A b) was remarkably less than the reduction of wild-type hSkM1 (Fig. 1). In contrast, after reduction with 1 mM DTT, the decrease in current through Y401C+E758C  $\mu$ 1 by 1  $\mu$ M Hg<sup>2+</sup> (Fig. 5 A c) was much greater than the decrease in hSkM1. Fig. 5 B indicates the relationship between Hg<sup>2+</sup> dose and the reduction of current either through the Y401C+E758C  $\mu$ 1 or through wild-type hSkM1. Y401C+E758C  $\mu$ 1 (before reduction) had significantly lower affinity for Hg<sup>2+</sup> (1  $\mu$ M,  $5.6 \pm 12.3\%$ ,  $n = 9$ ; 3  $\mu$ M,  $3.4 \pm 8.9\%$ ,  $n = 8$ ; 10  $\mu$ M,  $25.9 \pm 31.6\%$ ,  $n = 7$ ;  $p < 0.01$ ) than wild-type hSkM1 (1  $\mu$ M,  $53.7 \pm 17.3\%$ ,  $n = 6$ ; 3  $\mu$ M,  $83.2 \pm 13.1\%$ ,  $n = 5$ ; 10  $\mu$ M, 100%,  $n = 4$ ). However, Y401C+E758C  $\mu$ 1 after reduction had the higher affinity for Hg<sup>2+</sup> (1  $\mu$ M, 100%; 3  $\mu$ M, 100%; 10  $\mu$ M, 100%;  $n = 4$ ). According to the calculated equation (Eq. 1), the  $K_d$  value of Y401C+E758C  $\mu$ 1 before reduction was 18  $\mu$ M with a Hill coefficient value of 1.02,



**FIGURE 5** Block of the double mutant Y401C+E758C  $\mu$ 1 by  $\text{HgCl}_2$  in comparison with that of wild hSkM1 by  $\text{HgCl}_2$ . (A) Representative current traces (a) indicating the original current of Y401C+E758C before ( $\circ$ ) and during ( $\bullet$ ) treatment with 1 mM DTT. DTT increased the Y401C+E758C  $\mu$ 1 current. (b) Representative traces indicating the original current traces of Y401C+E758C  $\mu$ 1 in the absence ( $\circ$ ) and presence ( $\bullet$ ) of 3  $\mu\text{M}$  and the presence of 10  $\mu\text{M}$   $\text{Hg}^{2+}$  ( $\blacksquare$ ).  $\text{Hg}^{2+}$  (10  $\mu\text{M}$ ) blocked Y401C+E758C  $\mu$ 1 slightly. (c) Representative traces indicating the original current traces of Y401C+E758C  $\mu$ 1 (after reduction with DTT) in the absence ( $\circ$ ) and presence ( $\bullet$ ) of 1  $\mu\text{M}$   $\text{Hg}^{2+}$ .  $\text{Hg}^{2+}$  (10  $\mu\text{M}$ ) completely blocked Y401C+E758C  $\mu$ 1. (B) The relationship between the reduction of Y401C+E758C  $\mu$ 1, before reduction ( $\square$ ) or after reduction ( $\blacksquare$ ), or of wild hSkM1 and  $\text{Hg}^{2+}$  ( $\square$ ). Each column plus bar indicates the mean  $\pm$  SD value of the % block of wild hSkM1 and Y401C+E758C  $\mu$ 1 before and after reduction at various  $\text{Hg}^{2+}$  concentrations.



but for Y401C+E758C  $\mu$ 1 after reduction  $K_d$  was 0.36  $\mu\text{M}$  with a Hill coefficient value of 1.45. These results suggested that closely spaced free sulfhydryl groups may covalently bond as a disulfide bridge and thus render cysteinyl side chains unavailable to bind  $\text{Hg}^{2+}$ .

### The modification of conserved cysteinyl residues in the P-loop region influences $\text{Hg}^{2+}$ -induced block

There are two conserved cysteinyl residues between skeletal muscle and heart in the P-loop region, i.e., C753 of domain II and C1521 of domain IV. To identify the binding site for determinants for  $\text{Hg}^{2+}$  block of skeletal muscle-type  $\text{Na}^+$  channel, we studied the effects of  $\text{Hg}^{2+}$  on mutant C753A $\mu$ 1 or C1521A  $\mu$ 1. As shown in Fig. 6 A, 10  $\mu\text{M}$   $\text{Hg}^{2+}$  blocked C1521A  $\mu$ 1 by only 60%. Fig. 6 B indicates the summary of the effects of  $\text{Hg}^{2+}$  at various concentrations (0.01–60  $\mu\text{M}$ ) on either C753A  $\mu$ 1 or C1521A  $\mu$ 1 in comparison with wild-type hSkM1. The  $K_d$  of C753A  $\mu$ 1 was 8.47  $\mu\text{M}$  with a Hill coefficient of 2.89, and the  $K_d$  of C1521A  $\mu$ 1 was 8.63  $\mu\text{M}$  with a Hill coefficient of 2.90.  $\text{Hg}^{2+}$ -induced block of either C753A  $\mu$ , or C1521A  $\mu$ 1 was thus significantly smaller than that of wild-type hSkM1. These results suggest that the two conserved cysteinyl residues of the P-loop region play an important role in the block of the wild-type skeletal muscle-type  $\text{Na}^+$  channel  $\alpha$  subunits by  $\text{Hg}^{2+}$ .

### The high sensitivity of hH1 to $\text{Hg}^{2+}$ compared to hSkM1

Fig. 7 A demonstrates the effects of  $\text{Hg}^{2+}$  at 0.1 and 0.3  $\mu\text{M}$  on both hSkM1 (a and c) and hH1 (b and d). Although low  $\text{Hg}^{2+}$  had only a slight inhibitory effect on hSkM1 (0.1  $\mu\text{M}$ , 0%; 0.3  $\mu\text{M}$ , 6%), it had a marked inhibitory effect on hH1 (0.1  $\mu\text{M}$ , 20%; 0.3  $\mu\text{M}$ , 50%). Fig. 7 B indicates the relationship between the ratio of the blockages of hSkM1 and hH1 and the concentrations of  $\text{Hg}^{2+}$  obtained from four to six experiments. The lower concentrations of  $\text{Hg}^{2+}$  (in the range of 0.1 and 0.3  $\mu\text{M}$ ) significantly and selectively blocked hH1 (0.1  $\mu\text{M}$ ,  $25 \pm 23\%$ ; 0.3  $\mu\text{M}$ ,  $42 \pm 17\%$ ;  $p < 0.05$ ) but did not appreciably block hSkM1 (0.1  $\mu\text{M}$ , 0%; 0.3  $\mu\text{M}$ ,  $7 \pm 8\%$ ). According to Eq. 1, the  $K_d$  and Hill coefficient for  $\text{Hg}^{2+}$  were significantly different for hH1 (0.43  $\mu\text{M}$  and 0.93) versus hSkM1 (0.96  $\mu\text{M}$  and 1.84). These results show that the sensitivity of hH1 for  $\text{Hg}^{2+}$  is significantly greater than that of the hSkM1 channel and implies that sites of block of the two channels by  $\text{Hg}^{2+}$  might be different.

### Cysteine<sup>373</sup> is responsible for the high sensitivity of hH1 to the low dose of $\text{Hg}^{2+}$

A naturally variant cysteine in the pore region in domain I of the  $\text{Na}^+$  channel is present in hH1 (Cys<sup>373</sup>) but absent from hSkM1 (Tyr<sup>407</sup>). Therefore, we hypothesized that Cys<sup>373</sup> is the residue responsible for the increased sensitiv-

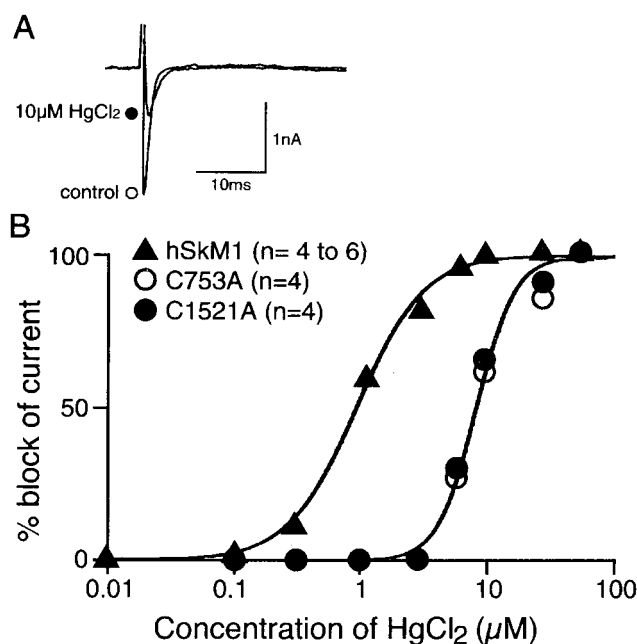


FIGURE 6 Blocking effects of HgCl<sub>2</sub> on C753A $\mu$ 1 and C1521A $\mu$ 1 in comparison with that on hSkM1. (A) Representative current traces of C1521A $\mu$ 1.  $\circ$ , C1521A $\mu$ 1 in control.  $\bullet$ , C1521A $\mu$ 1 in the presence of 10  $\mu$ M Hg<sup>2+</sup>. Hg<sup>2+</sup> (10  $\mu$ M) blocked C1521A $\mu$ 1 by only 60%. (B) The mean values of the % block of Hg<sup>2+</sup> on C753A $\mu$ 1 ( $\circ$ ) and C1521A $\mu$ 1 ( $\bullet$ ) induced by Hg<sup>2+</sup> in comparison with that of hSkM1 ( $\blacktriangle$ ) at various concentrations of Hg<sup>2+</sup> (0.01–60  $\mu$ M) as obtained ( $\bullet$ ) from four to six individual experiments. The solid lines show the best fit by Eq. 1 with the optimal values of the parameters.

ity of hH1 to the lower dose of Hg<sup>2+</sup>. Fig. 8 A indicates the effects of Hg<sup>2+</sup> at 0.1 (a), 0.3 (b), and 1.0  $\mu$ M (c) on the mutant C373Y hH1. The low dose of Hg<sup>2+</sup> produced a smaller block of C373Y hH1 (0.1  $\mu$ M, 0%; 0.3  $\mu$ M, 3%; 1.0  $\mu$ M, 19%) than that of wild-type hH1, as shown in Fig. 7. Fig. 8 B is a plot of the relationship between the ratio of the blockage of the C373Y hH1 and the wild-type hH1 and the concentrations of Hg<sup>2+</sup> obtained from four to six experiments. Lower concentrations of Hg<sup>2+</sup> (in the range of 0.1 and 0.3  $\mu$ M) did not sufficiently block C373Y hH1 (0.1  $\mu$ M, 0%; 0.3  $\mu$ M, 11.4  $\pm$  12%;  $p$  < 0.05) in comparison with wild-type hH1 (0.1  $\mu$ M, 25  $\pm$  23%; 0.3  $\mu$ M, 42  $\pm$  17%). According to Eq. 1, the  $K_d$  and Hill coefficient for Hg<sup>2+</sup> were significantly higher for C373Y hH1 (1.12  $\mu$ M and 1.60, respectively) than for wild-type hH1 (0.430  $\mu$ M and 0.93, respectively).

## DISCUSSION

In the present report we studied the effects of sulfhydryl-oxidizing agents on the function of the  $\alpha$  subunit of Na<sup>+</sup> channels expressed in COS7 cells. We demonstrated that 1) the sulfhydryl-oxidizing compound Hg<sup>2+</sup> but neither thimerosal nor DTDP modified hSkM1, resulting in a re-

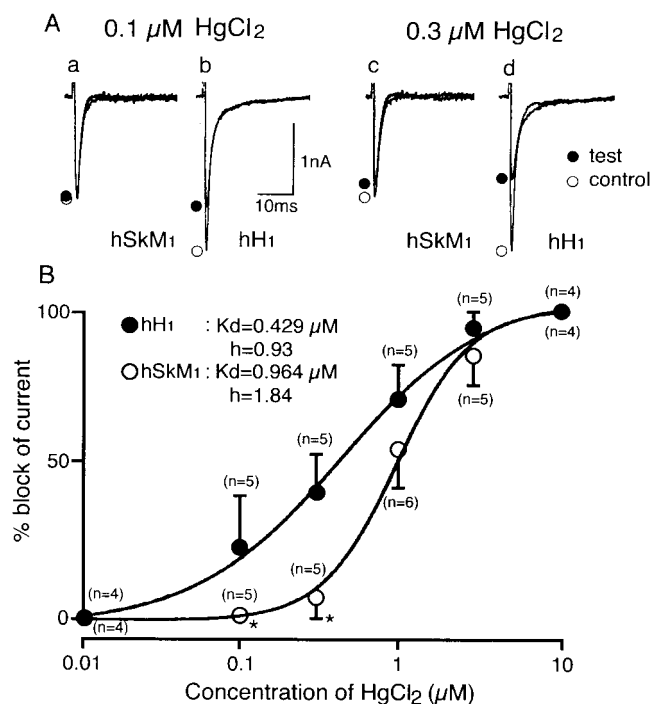


FIGURE 7 Blocking effect of HgCl<sub>2</sub> on hH1 in comparison with that on hSkM1. (A) Representative current traces of hSkM1 (a and c) and hH1 (b and d).  $\circ$ , hSkM1 and hH1 in the absence of Hg<sup>2+</sup>.  $\bullet$ , hSkM1 and hH1 in the presence of 0.1 or 0.3  $\mu$ M Hg<sup>2+</sup>. (B) The mean  $\pm$  SD values of the % block of hH1 ( $\circ$ ) induced by Hg<sup>2+</sup> in comparison with that of hSkM1 ( $\bullet$ ) at various concentrations of Hg<sup>2+</sup> (0.1–10  $\mu$ M), as obtained from four to six individual experiments. The solid lines show the best fit by Eq. 1 with the optimal values of the parameters.

duction of  $\alpha$  hSkM1 current; however, pretreatment with thimerosal reduced the sensitivity of hSkM1 to Hg<sup>2+</sup>; 2) Hg<sup>2+</sup>-induced oxidation of the hSkM1 channel could be readily protected against or reversed by reducing agents such as DTT; 3) the Y401C+E758C  $\mu$ 1 mutant that forms a disulfide bond spontaneously demonstrated significantly lower sensitivity to Hg<sup>2+</sup> than wild-type hSkM1 but showed significantly higher sensitivity to Hg<sup>2+</sup> after reduction by DTT; 4) the C753A $\mu$ 1 and C1521A  $\mu$ 1 mutant demonstrated significantly lower sensitivity to Hg<sup>2+</sup> than wild hSkM1; 5) hH1 had significantly higher sensitivity to Hg<sup>2+</sup> than hSkM1; and 6) the C373Y mutant hH1 showed a significantly reduced sensitivity to Hg<sup>2+</sup> compared with wild-type hH1.

## The manner of oxidation by Hg<sup>2+</sup>

When Hg<sup>2+</sup> blocks sodium channels by oxidizing cysteinyl residues, Hg<sup>2+</sup> is known to bridge two sulfhydryl ligands (sulfur-Hg-sulfur) and is also known to complex with a single sulfhydryl group (sulfur-Hg<sup>+</sup>). The coordinated binding between Hg<sup>2+</sup> and ligands such as the sulfhydryl group generically exhibits the nature of a covalent bond rather

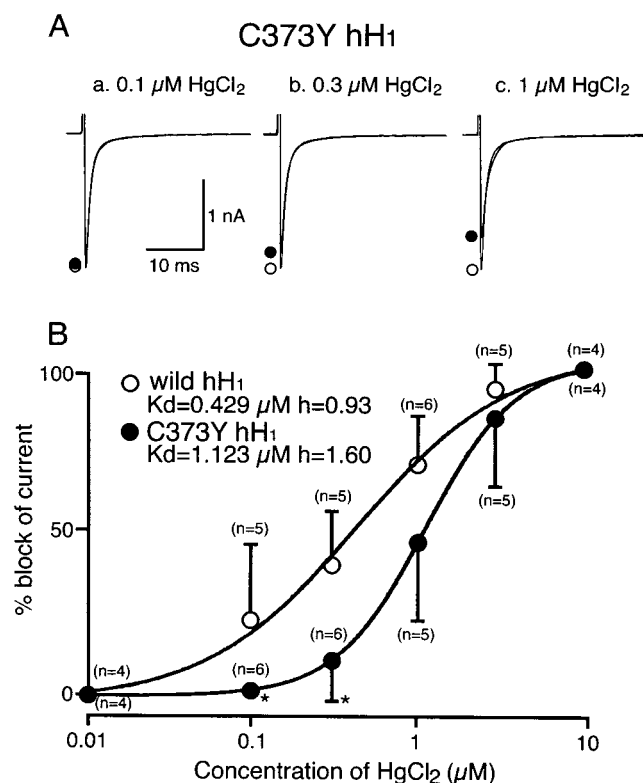


FIGURE 8 Block of the single mutant C373Y hH1 in comparison with that of wild hH1 by  $\text{Hg}^{2+}$ . (A) Representative traces indicating the C373Y hH1 before (○) and in the presence of 0.1 (a, ●), 0.3 (b, ●), and 10 μM (c, ●). (B) The mean value  $\pm$  SD of the % block of the wild hH1 (○) and the C373Y hH1 (●) at various concentrations of  $\text{Hg}^{2+}$  (0.1–10 μM) obtained from four to six experiments. The solid lines show the best fit by Eq. 1 with the optimal values of the parameters.

than an ionic bound. Especially when the coordination number (number of ligands) is 2, affinities to ligands of  $\text{Hg}^{2+}$  are much higher than those of other group IIB cations, such as  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  (Cotton and Wilkinson, 1980). Thus  $\text{Hg}^{2+}$  is likely to oxidize SH groups of cysteinyl residues on the Na channel protein by forming high-affinity Hg-S bonds (S-Hg-S).

### $\text{Hg}^{2+}$ blocks hSkM1 by oxidatively forming a coordinated ligation between the double cysteinyl residue pair

According to the general principles for the actions of sulfhydryl reagents (Ziegler, 1985), the present data are interpreted from the aspect of the interaction between the sulfhydryl modifier and multiple free sulfhydryl groups or native S-S bonds involving conformational changes in the ion channel proteins.  $\text{Hg}^{2+}$  blocked hSkM1 in a dose-dependent manner. Reduction with DTT protected hSkM1 from  $\text{Hg}^{2+}$ -induced block and produced  $\text{Hg}^{2+}$  insensitivity. DTT has been well established to specifically reduce S-S

bonds to free sulfhydryl groups. DTT may thus reduce S-S bonds in the  $\alpha$  subunit of the  $\text{Na}^+$  channel, affecting the  $\text{Hg}^{2+}$  sensitivity via conformational changes involving the  $\text{Hg}^{2+}$  binding site. In the minK channel the accessibility of  $\text{Hg}^{2+}$  to the channel has been reported to be dependent on the channel conformational change (Busch et al., 1995). Therefore, DTT-induced conformational change in hSkM1 could also produce inaccessibility of  $\text{Hg}^{2+}$  to the binding site, thereby affecting  $\text{Hg}^{2+}$  sensitivity. Moreover, block of hSkM1 by  $\text{Hg}^{2+}$  was quite stable in the absence of a reducing agent; however, application of DTT did partially reverse the effect of  $\text{Hg}^{2+}$ , as observed in other examples (Islam et al., 1993; Ruppersberg et al., 1991). These observations support the idea that  $\text{Hg}^{2+}$  blocks the  $\text{Na}^+$  channel by reacting with free sulfhydryl groups and/or by promoting the oxidation of adjacent free sulfhydryl groups. Other sulfhydryl-oxidizing agents such as thimerosal or DTDP did not block hSkM1, despite their sufficient capability to oxidize cysteinyl residues. The finding that hSkM1 was resistant either to DTDP or to thimerosal confirmed the report by Chiamvimonvat et al. (1995), showing that the sulfhydryl oxidation did not change hH1. The molecular size difference of thimerosal or DTDP versus  $\text{Hg}^{2+}$  might explain the different modification abilities of these sulfhydryl-oxidizing reagents. The smaller  $\text{Hg}^{2+}$  cation can access the cysteinyl residues located deep within a pocket in a region inaccessible to larger modifying agents such as DTDP or thimerosal. In fact, cysteinyl residues located >20% across the electrical field from the outside could not be modified by MTS agents, but could be modified by  $\text{Cd}^{2+}$  (Yamagishi et al., 1997). Alternatively, it is possible that a mixed disulfide is actually formed between thimerosal or DTDP and a target cysteinyl, but  $\text{Na}^+$  conductance is not changed, since T1235C $\mu$ 1 is modified by  $\text{Cd}^{2+}$  at a location ~20% across the electrical field but is not modified by MTSEA. In the present study, the pretreatment with thimerosal inhibited the degree of the  $\text{Hg}^{2+}$ -induced block of hSkM1, supporting the concept that thimerosal and other sulfhydryl modifiers can access the target cysteinyl residues but do not block the channel.

$\text{Hg}^{2+}$  could also promote oxidation in the form of coordinated ligation of the cysteinyl residue pair (a sulfur-Hg-sulfur bridge), but also oxidation to form sulfur-Hg<sup>+</sup> covalently. As shown in the present study, treatment with DTT increased the current amplitude of the Y401C+E758C  $\mu$ 1 mutant, indicating that a disulfide bond forms spontaneously between the flexible residue at the 401 and 758 positions of the Y401C+E758C  $\mu$ 1, as described by Benitah et al. (1996). Because disulfide formation depends entirely on the amount of motion that the residue undergoes, residues that are located in a rigid structure are less likely to form disulfides than residues located in a more flexible region (Tsushima et al., 1997). The Y401C+E758C  $\mu$ 1 showed a lower sensitivity to  $\text{Hg}^{2+}$  than wild-type hSkM1; however, after reduction the Y401C+E758C  $\mu$ 1 mutant

showed a higher sensitivity to Hg<sup>2+</sup> than did wild-type hSkM1. These results suggest that once a disulfide bond forms between the residue at the 401 and 758 positions, the same side chain would be unavailable for binding with Hg<sup>2+</sup>. Therefore, Hg<sup>2+</sup> can also promote oxidation, resulting in the formation of the coordinated ligation between the flexible cysteinyl residue pair (a sulfur-Hg-sulfur bridge) through the aqueous pathway. The double mutant Y401C+E758C  $\mu$ 1 and Y401C+G1530C  $\mu$ 1 had previously been reported to produce a bridge metal coordination site for Cd<sup>2+</sup> and Zn<sup>2+</sup> (Benitah et al., 1996, 1997; Tomaselli, 1997). Hg<sup>2+</sup> thus appears to form a similar coordinated ligation involving the cysteinyl residue pair, which shares the metal coordination site with the other thiol-avid group IIB divalent cations. However, the present data do not exclude the possibility that Hg<sup>2+</sup> oxidizes sulfhydryl groups to form sulfur-Hg<sup>+</sup> with covalent character, leading to a block of the hSkM1 channel. Because there are two conserved cysteinyl residues in the P-region of wild-type hSkM1 or wild-type  $\mu$ 1 channels, only one cysteinyl residue exists in the P-region of the C753A  $\mu$ 1 and C1521A  $\mu$ 1 mutants. In the present study, C753A  $\mu$ 1 and C1521A  $\mu$ 1 mutants exhibited significantly lower sensitivity to Hg<sup>+</sup> than did wild-type hSkM1. However, Hg<sup>+</sup> does block C753A  $\mu$ 1 and C1521A  $\mu$ 1. This suggests that Hg<sup>2+</sup> can oxidize sulfhydryl groups to form sulfur-Hg<sup>+</sup> covalently and thus block  $\mu$ 1 channels, but the sulfur-Hg<sup>+</sup> covalent bond may be less effective in blocking the channel than the sulfur-Hg-sulfur mechanism.

### Comparison block by Hg<sup>2+</sup> with previous studies of thiol-avid group IIB divalent cations

Cd<sup>2+</sup>, Zn<sup>2+</sup>, and Hg<sup>2+</sup> belong to the thiol-avid group IIB divalent cations. Chemical modification experiments (Schild et al., 1991; Doyle et al., 1993) or mutant substitution experiments (Benitah et al., 1996, 1997; Tomaselli, 1997) indicate that Cd<sup>2+</sup> and Zn<sup>2+</sup> interact with the unique sulfhydryl group of the heart Na<sup>+</sup> channel at the same metal coordination sites demonstrated for Hg<sup>2+</sup> in the present study. The cysteinyl residues participating in the metal coordination site place the  $\alpha$  carbons within 3.8–6.8 Å of each other (Srinivasan et al., 1990). The fact that in the double mutant Y401C+E758C  $\mu$ 1 disulfide bond forms spontaneously and reforms spontaneously soon after being disrupted suggested an average separation among cysteinyl residues in this pair of no greater than 5 Å (Perry and Wetzel, 1986; Pantoliano et al., 1987; Falke et al., 1988). Based on this information, the distance between the cysteinyl residue pair, which Hg<sup>2+</sup> can ligate oxidatively, is estimated to be less than 5 Å. Whereas either the Cd<sup>2+</sup>- or Zn<sup>2+</sup>-induced block of Na<sup>+</sup> channel  $\alpha$  subunit can readily be restored by simple washout (data not shown), the Hg<sup>2+</sup>-induced block of the Na<sup>+</sup> channel  $\alpha$  subunit was stable after Hg<sup>2+</sup> washout in the absence of a reducing agent. The  $K_d$

value at micromolar concentration for Hg<sup>2+</sup>-induced block of the muscle Na<sup>+</sup> channel  $\alpha$  subunit was markedly lower than the millimolar  $K_d$  values for Cd<sup>2+</sup> or Zn<sup>2+</sup>. These results indicate that Hg<sup>2+</sup> strongly oxidizes the free SH groups to form sulfur-Hg-sulfur, resulting in the block of the Na<sup>+</sup> channel  $\alpha$  subunit; in contrast, Cd<sup>2+</sup> and Zn<sup>2+</sup> bind to the free thiols semicovalently. There are two naturally occurring cysteinyl residues in domains II and IV in the P-loop region of hSkM1, which may be the potential target for Hg<sup>2+</sup>. According to the topology of the P-loop region in the Na<sup>+</sup> channel pore revealed by cysteine mutagenesis (Yamagishi et al., 1997), these cysteinyl residues in the P-loop regions of domains II and IV could not be modified by Cd<sup>2+</sup>, suggesting that these cysteinyl residues are buried in the protein. However, Hg<sup>2+</sup> can effectively block hSkM1. It is possible that Hg<sup>2+</sup> can modify these two naturally occurring cysteinyl residues in domains II and IV of hSkM1 because of its higher degree of oxidation and its reaction sphere, which is larger than that of Cd<sup>2+</sup>. In fact, C753A  $\mu$ 1 and C1521A  $\mu$ 1 have exhibited reduced sensitivity to Hg<sup>2+</sup>, suggesting that Hg<sup>2+</sup> has access these two conserved cysteinyl residues in the P-loop region through a hydrophilic pathway and block  $\mu$ 1 channels. In addition, the pretreatment with thimerosal reduced the sensitivity of hSkM1 to Hg<sup>2+</sup>, suggesting that thimerosal has access to two conserved cysteinyl residues in the P-loop region of hSkM1. These results indicate that two conserved residues in the P-region are not buried in the protein, but they are accessible from the outside. As these conserved cysteinyl residues in the P-loop region can be modified by the Hg<sup>2+</sup> and the thimerosal but not by the thiol-avid group of Cd<sup>2+</sup> and Zn<sup>2+</sup> at micromolar concentrations, Hg<sup>2+</sup> can block hSkM1 at micromolar affinity, but either Cd<sup>2+</sup> or Zn<sup>2+</sup> can block hSkM1 at millimolar affinity.

### Different sensitivities of hH1 and hSkM1 to sulfhydryl-oxidizing compound

It is interesting to note that in the present experiments, the hH1 channel was intrinsically more sensitive to blocking by Hg<sup>2+</sup> than was hSkM1. A naturally variant cysteine in the pore of the Na<sup>+</sup> channel, present in the cardiac isoform (Cys<sup>373</sup>) but absent from the skeletal muscle (Tyr<sup>407</sup>), has been reported to influence both the Na<sup>+</sup> flux and the block of Na<sup>+</sup> flux by the thiol-avid group IIB divalent cations, guanidinium toxins, tetrodotoxin, and saxitoxin (Satin et al., 1992; Baines et al., 1997). Therefore, the single cysteinyl residue within the pore-forming region of domain I might be responsible for the different sensitivities to Hg<sup>2+</sup> of the hH1 and hSkM1 channels. The finding that C373Y hH1 had significantly less sensitivity to the low dose of Hg<sup>2+</sup> compared to the wild-type hH1 but almost the same sensitivity as hSkM1 demonstrated that the heart-specific sensitivity to the low dose of Hg<sup>2+</sup> could be due, at least partly, to the presence of the cysteine residue of the pore-forming region



in domain I of hH1. Based on data on Y401C+E758C  $\mu$ 1, a naturally variant cysteine in hH1 that is localized to 20% of the distance between the electrical field and is adjacent to the proposed selective filter of the channel (Backx et al., 1992) is considered to be located in a more flexible region.

It is still unknown which cysteinyl residues in the P-loop region may form paired coordination sites for  $\text{Hg}^{2+}$  with Cys<sup>373</sup> in hH1. Benitah et al. (1996) reported that by means of paired cysteine substitution experiments, domain III was the only one that failed to reveal any interactions of mutated P-loop residues with Y401C, while domains II and IV formed a spontaneous disulfide bond or the metal coordinated site with Y401C, respectively. In the present study we showed that  $\text{Hg}^{2+}$  can react both to the cysteinyl residue in the domain II (C753) and to the cysteinyl residue in the domain IV (C1521). Taken together, it may be suggested that interaction between the flexible cysteine in the P-loop region of domain I and the cysteinyl residue of the presumed lateral neighbors of either the domain II or the domain IV can occur readily. Thereby, in heart,  $\text{Hg}^{2+}$  may oxidatively create a coordinated ligation between domains I and II or IV of the  $\text{Na}^+$  channel  $\alpha$  subunit. In fact, the  $K_d$  values for Y401C+E758C  $\mu$ 1 after reduction, wild-type hH1, wild-type hSkM1, C373YhH1, Y401C+E758C  $\mu$ 1 before reduction, C753A  $\mu$ 1, and C1521A  $\mu$ 1 were 0.36, 0.43, 0.96, 1.12, 18, 8.47, and 8.63  $\mu\text{M}$ , respectively. The number of the cysteinyl residues in the P-region was four in Y401C+E758C  $\mu$ 1 after reduction; three in wild-type hH1; two in wild-type hSkM1, C373YhH1, and Y401C+E758C  $\mu$ 1 before reduction; and one in C753A  $\mu$ 1 and C1521A  $\mu$ 1. These results may suggest that the channels with more than one cysteinyl residue in the P-loop region have a higher sensitivity to  $\text{Hg}^{2+}$  ( $K_d < 1.12 \mu\text{M}$ ) than the channels with the single cysteinyl residue in the P-loop region ( $K_d > 8.47 \mu\text{M}$ ), except for Y401C+E758C  $\mu$ 1 before reduction. These findings also suggest that the sulfur-Hg-sulfur bridge between the cysteinyl residue of the domain I and the other conserved cysteinyl residues in the P-loop region would determine the higher sensitivity to  $\text{Hg}^{2+}$  with the smaller  $K_d$  value, and the sulfur- $\text{Hg}^+$  covalent bond at the conserved cysteinyl residue in the P-loop region would determine the block of  $\text{Hg}^{2+}$  with the larger  $K_d$  value. The Hill coefficient of  $\text{Hg}^{2+}$  block of Y401C+E758C  $\mu$ 1 after reduction and hH1 significantly differs from those of hSkM1, C373Y hH1, Y401C+E758C  $\mu$ 1 before reduction, C753A  $\mu$ 1, and C1521A  $\mu$ 1. The value of the Hill coefficient was close to 1.0 in Y401C+E758C  $\mu$ 1 after reduction and hH1, but it was more than 1.0 in hSkM1, C373Y hH1, Y401C+E758C  $\mu$ 1 before reduction, C753A  $\mu$ 1, and C1521A  $\mu$ 1. The relatively high Hill coefficient for  $\text{Hg}^{2+}$  block of hSkM1, C373Y hH1, Y401C+E758C  $\mu$ 1 before reduction, C753A  $\mu$ 1, and C1521A  $\mu$ 1 may reflect the multiple binding of  $\text{HgCl}_2$  molecules to cysteine residues, which is different from the observations for other thiol-avid group IIb divalent cations. It is known that  $\text{HgCl}_2$  can produce a long bridge

between two SH groups; for instance, two  $\text{HgCl}_2$  molecules possibly form a  $\text{S}(\text{HgCl}_2)_2\text{S}$  bridge. One  $\text{Hg}^{2+}$  molecule might bind to the cysteine residues of Y401C+E758C  $\mu$ 1 after reduction and to hH1 reflecting a Hill coefficient of  $\sim 1.0$ , although multiple  $\text{HgCl}_2$  molecules might bind to the cysteinyl residues of hSkM1, C373Y hH1, Y401C+E758C  $\mu$ 1 before reduction, C753A  $\mu$ 1, and C1521A  $\mu$ 1, reflecting a Hill coefficient greater than 1.0. However, further experiments will be necessary to clarify this hypothesis.

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